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The purpose of this project is to determine whether women who developed bilateral breast cancer following radiotherapy for an initial breast cancer had a higher incidence of heterozygosity for a mutation in the *ATM* gene than either breast cancer patients who did not develop a second breast cancer or bilateral breast cancer patients who did not receive radiotherapy. The approach has been to amplify with PCR the *ATM* gene in segments employing either cDNA produced using RNA isolated from blood lymphocytes or DNA extracted from paraffin-embedded biopsy tissue samples. During the first year of this project a total of 115 tissue samples from unilateral and bilateral breast cancer patients have been obtained, PCR products produced from these samples and examined for mutations using a Non-radioisotopic RNase Cleavage-based Assay (NIRCA). Analysis of the entire *ATM* coding region has been completed for 29 unilateral breast cancer patients and no mutations were identified. These results are consistent with the conclusion that the *ATM* mutation rate among unilateral breast cancer patients is no greater than the 0.5-1% observed for the general population. Work is now in progress to perform this mutational analysis for bilateral breast cancer patients.

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Roger S. Rosato 7/9/98

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INTRODUCTION

Breast cancer is the most common form of cancer among women in the United States (Wingo 1995) and it is well recognized that women with proven cancer in one breast are at increased risk of developing a second malignancy in the contralateral breast (Prior 1978, Horn 1987, Donovan 1990, Bernstein 1992a,b). The rising incidence of breast cancer coupled with an improved survival potential after cancer diagnosis has placed an increased number of women at risk for second primary breast cancer.

The gene, *ATM*, that is mutated in the autosomal recessive disorder ataxia telangiectasia (AT) was identified by positional cloning on chromosome 11q22-23 (Savitsky 1995). It encodes a 12 kb transcript from which a putative protein of 3056 residues is synthesized (Savitsky et al., 1995) which is similar to several yeast and mammalian phosphatidylinositol-3'kinases that are involved with signal transduction and cell cycle control. AT is a distinctive autosomal recessive syndrome characterized by an increase cancer incidence, progressive cerebral ataxia and oculocutaneous telangiectasias (Boder 1985, Swift 1987). Patients with AT usually have chromosomal abnormalities, severe deficiencies in the humoral and cellular immune responses, growth retardation, premature aging, and increased sensitivity to ionizing radiation and radiomimetic chemicals (Lehman 1982, Spector 1982, Savitsky 1995).

AT, which results from mutations in both copies of the *ATM* gene, is relatively rare with an incidence of approximately only 1 in 100,000 (Sedgwick and Boder, 1991). However, the heterozygous condition which is characterized by a mutation in one copy of the gene, is thought to affect roughly 0.5-1% of the population (Swift et al. 1986). These individuals do not suffer from AT, however, they appear to be particularly susceptible to the induction of breast cancer by radiation (Swift 1991). The hypothesis being examined in this project is that women who are *ATM* gene carriers and receive radiation therapy, generally as part of a breast conservation treatment, are at increased risk of developing a second primary breast cancer. Hence, the goal of this study is to determine whether radiation exposure resulting from the scattered dose to the contralateral breast (Boice et al., 1979; Hankey et al., 1983; Basco et al., 1985; Fraas et al., 1985; Kurtz et al., 1988; Harris and Coleman, 1989; Kelly et al., 1996) is associated with an increased occurrence of bilateral breast cancer among women who are heterozygous for *ATM*.

Confirmation of this hypothesis would have important and direct implications upon patient care in that it would suggest that breast cancer patients should be tested for *ATM* heterozygosity. If found positive, a possible recommendation may

be that these women should not be candidates for radiotherapy and other treatment modalities utilized. Alternatively, a more effective block technique with dose reduction to the contralateral breast may be implemented for these patients. This would have the positive impact upon the vast majority of patients that are not *ATM* carriers as they could be assured that their risk for radiation induced breast cancer is extremely low as *ATM* carriers may account for most breast cancers resulting exposure of the contralateral breast to radiation associated with radiotherapy.

This study could have important implications for the general population as well, in that it may suggest that women prior to mammography be tested for *ATM* heterozygosity and that individuals who are positive should be examined with a diagnostic modality not involving use of ionizing radiation. This approach may greatly reduce the small, but significant, number of breast cancers which are induced by this radiation exposure. In addition, this would assure the estimated 99% of women who are not carriers, that the radiation doses associated with mammography are of little biologic hazard to them.

BODY

Task 1: Month 1: Identify with Dr. Paul Tartter the bilateral breast cancer patients who fall into the two categories to be used in this study.

This was accomplished using records Dr. Tartter has assembled for bilateral breast cancer patients treated at Mount Sinai over the past 18 years.

Task 2: Month 1: Establish a procedure by which the breast cancer patients will be recruited into the study.

There was relatively easy accessibility to unilateral breast cancer patients as many of these patients were treated in the Radiation Oncology Department and continue to come to Mount Sinai periodically for follow-up visits. Arrangements were made so that when patients selected for this study arrived for their exam, an investigator was available to explain the project to the patients and obtain informed consent for participation in the study following which a nurse would draw a blood sample. A difficulty arose, however, recruiting bilateral breast cancer patients into the study as many of these patients, who were treated as long as 18 years ago, had either died, been lost to follow up or rarely visited Mount Sinai. Therefore, a different strategy was developed to access the bilateral patients which was to obtain paraffin embedded tissue, preferably lymph node biopsy samples from these individuals. A collaboration was established with Dr. Ira Bleiweiss, the pathologist at Mount Sinai responsible for review of all breast cancers. Using this approach, we have successfully retrieved paraffin-embedded tissue blocks from 63 of the 69 bilateral patients that Dr. Tartter identified.

Task 3: Month 1: Decide which regions of ATM will be subjected to analysis based upon results available in the literature at the time the project begins

As of this point, there are no specific allelic hotspots or regions of *ATM* which have been identified for mutations. Therefore, the entire coding region of the gene is being examined.

Task 4: Months 2-32: Obtain blood samples from breast cancer patients

Blood samples have been obtained from a total of 52 unilateral breast cancer patients. In addition, tissue blocks have been retrieved for 63 bilateral breast cancer patients.

Task 5: Month 2: Create appropriate primers for RT-PCR and sequencing

During the period when a procedure to recruit bilateral breast cancer patients into the study was being established, efforts were also initiated to create primers for RT-PCR. As this effort was well underway by the time a decision was made to use genomic DNA obtained from paraffin embedded tissue, it was decided to complete construction of the cDNA primers and to use them for unilateral breast cancer patients. The primers which were designed and validated are listed in Tables 1 and 2.

Work is also in progress to design and validate primers to amplify all 66 of the exons which comprise the *ATM* gene with 22 sets of primers created at this point. It is expected that in many cases it will be possible to combine two exons into a single fragment for those instances in which the intervening intron is no longer than approximately 1 kb. Therefore, it should be possible to amplify the 66 *ATM* exons in roughly half this number of PCR reactions.

Task 6: Months 3-36: Isolate mRNA from blood lymphocytes of patients

During the past year, a total of 52 breast cancer patients were recruited into this study and RNA and DNA isolated from blood lymphocytes obtained from these patients. Lymphocytes were first isolated by layering 4 ml of blood on 3 ml FICOL which was centrifuged for 20 min and the lymphocyte layer collected. The cells were washed three time with 4° PBS and 2 ml of RNAzol™ B added to half of the sample. 200 µl of chloroform was added, kept on ice 5 min and centrifuged at 12,000 rpm (4°) for 15 min. The upper aqueous phase was transferred to a fresh tube, an equal volume of isopropanol added and held for 15 min at 4°. The sample was centrifuged 15 min at 12,000 rpm (4°) and the precipitated RNA pelleted. The supernatant was removed, the RNA pellet washed once with 75% ethanol, air dried and dissolved in 1 mM EDTA, pH 7.

To the remaining half of the lymphocyte sample, DNA was extracted by adding 1 ml DNA STAT-60™ and the cells homogenized by pipetting the lysate several times. 200 µl chloroform was added, vortexed 15 sec and incubated at room temperature for 2-3 min. The homogenate was centrifuged at 12,000g 15

min (4°) and the upper aqueous phase containing DNA transferred to a fresh tube and mixed with 500 µl of isopropanol. The sample was held at room temperature 5-10 min and the DNA pelleted by centrifugation at 12,000g for 10 min at 4°. The supernatant was removed, the DNA pellet washed once with 75% ethanol, air dried and dissolved in 1 mM EDTA, pH7.

Efforts have recently begun to isolate genomic DNA from paraffin-embedded tissue from bilateral breast cancer patients. To accomplish this, the ONCOR EX-WAX™ DNA Extraction Kit is being used. The procedure has been to first cut 10 sections of 5 µ thickness from a paraffin-embedded lymph node biopsy sample and place them in a 1.5 ml tube. 1 ml of 100% ethanol was added, vortexed 15 sec, centrifuged at 12,000 rpm 3 min, the ethanol removed and the pellet air dried. 150 µl of digestion solution and 50 µl of protein digesting enzyme solution were added and the tube incubated 4-18 hr at 50°. 100 µl of extraction solution was mixed and after 15 sec the tube was centrifuged 10 min at 12,000 rpm. The supernatant was removed by poking the pipette tip through the paraffin layer on top and withdrawing the supernatant, leaving the paraffin and pellet behind. 150 µl of precipitation solution was added to the supernatant, the tube inverted 3 times, 900 µl of 100% ethanol (-20°) added and mixed. After 1 hr the tube was centrifuged for 10 min at 12,000 and the supernatant discarded. The pellet was air dried and 50 µl of resuspension solution added for 1 hr at 50°.

Task 7: Months 3-36: Perform RT-PCR with these samples to amplify in sections the coding region of the *ATM* gene.

The entire *ATM* coding region was amplified from 29 patients using the primers indicated in Tables 1 and 2. The procedure used was to first produce cDNA by mixing 1-5 µg total RNA with 1 µl oligo dT (500 µg/ml), H_2O to 12 µl and heating to 70° for 10 min and followed by chilling on ice. To this 4 µl 5X buffer, 2 µl 0.1 M DTT and 1 µl 10 mM dNTP mix were added and incubated at 42° for 2 min. 1 µl (200 units) SUPERSCRIPT™ II was added, the tube incubated 50 min at 42° and the reaction stopped by heating at 70° for 15 min. 1 µl (2 units) of RNase H was added to the tube and incubated for 20 min at 37°.

The first stage PCR reactions were performed using 10 µl cDNA (approximately 100-500 ng genomic DNA), 125 µM dNTPs, 250 nM primers, 1 U Taq polymerase in a buffer containing 10 mM Tris.Cl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin and RNase free water to a total volume of 50 µl. The PCR reactions were performed in a Perkin Elmer 4800 Thermal Cycle at 94° for 4 min, followed by 30 cycles of 1 min at 94°, 2 min at 54° and 1.5 min at 72° and one cycle for 8 min at 72°. 10 µl of the first stage PCR product was used in a second

series of PCR reactions using the same conditions to achieve further amplification of the region of interest and to add the T7 and SP6 promoters to the PCR products.

Task 8: Months 3-36: Use NIRCA to identify PCR products containing mutations

The 29 patients for whom the coding region was amplified using RT-PCR have been examined for mutations using a *N*on-radio*i*sotopic *R*Nase *C*leavage-based *A*ssay (NIRCA) (Myers et al., 1985; Winter et al., 1985; Ambion, 1996). Each second stage PCR product was transcribed in two reaction mixes using 2 µl of the PCR product, 250 nM rNTPs, 2 U of either T7 or SP6 RNA polymerase in a buffer containing 40 mM Tris.Cl, pH 7.5, 7 mM $MgCl_2$, 2 mM spermidine, 25 mM NaCl and 10 mM dithiothreitol and brought to a final volume of 10 µl with RNase free H_2O and incubated for 1 hr at 37°. 10 µl of buffer containing 80% (v/v) formamide, 25 mM NaCl, 2 mM EDTA, pH 8.0 was added to each transcription reaction. The T7 and SP6 reactions were mixed, incubated at 95° for 3 min and allowed to cool at room temperature for 1 min. 4 µl of the duplex reactions were mixed with 16 µl of RNase digestion buffer (10 mM NaCl, 10 mM Tris.Cl, pH 7.5, 1 mM disodium EDTA, pH 8.0) with 0.5 µg/ml RNase A, RNase I and RNase T1 and brought to a final volume of 20 µl. The reaction mixtures were incubated at 37° for 20 min and resolved on a 3% agarose gel run at 100 V for approximately 45 min.

The same procedure as described above is also being used for analysis of genomic DNA obtained from the paraffin embedded tissue derived from the bilateral breast cancer patients. In addition, several samples of lymphocyte DNA are being examined using the genomic DNA primers to confirm the results obtained using cDNA derived from lymphocyte RNA.

Task 9:Months 3-36: Sequence all PCR products which appear to exhibit mutations

There was evidence of a mutation in three samples obtained from unilateral breast cancer patients as indicated by the presence of multiple bands in the RNase digested samples. For these samples, the PCR products were isolated form a low melt agarose gel and the DNA isolated using a QIAquick Gel Extraction Kit and the DNA sequenced in an ABI automated sequencer. However, no mutations were confirmed through sequencing. Hence, at this point, no mutations in *ATM* have been identified and it would therefore appear from this work that the incidence of *ATM* mutations in breast cancer patients is relatively low and may be not higher than the approximately 0.5-1% observed for the general population. This is consistent with previously reported results (Vorechovsky et al., 1995).

TABLE 1. SEQUENCES OF FIRST STAGE *ATM* PRIMERS

Fragment Number	Bases Amplified	Primer Sequence
1-Sense	5-906	GAGGAGTCGGGATCTGC
1-Antisense		GACAGCCAAAGTCTTGAG
2-Sense	695-1676	CTGTGTACTTCAGGCTCT
2-Antisense		GCTTGTATTTGCTCAGAA
3-Sense	1544-2509	CACCATATGTGTTACGAT
3-Antisense		TCATATTTCTCAAGGAAC
4-Sense	2329-3298	ACTCTTGTCCGGTGTTC
4-Antisense		AGGGCCATTCTTACAGA
5-Sense	2978-3977	TAATTGATTCTAGCACGC
5-Antisense		CTTCTAATCACCAGATGT
6-Sense	3877-4850	AACTTATCTTCTTTTCC
6-Antisense		AATCGTGATATAGAGGT
7-Sense	4710-5682	TTACTGTAAGGATGCTC
7-Antisense		AGTCAGTTTTCACTTCA
8-Sense	5559-6534	TGATATAAATCTGTGGA
8-Antisense		TTCTACTTCTTTGCTG
9-Sense	6407-7353	TCTGCCATATTCTTTCC
9-Antisense		AACCGGGCTAATGAG
10-Sense	7193-8172	CAGAATGTCTGAGGGT
10-Antisense		TAGTAGGGACAACAACA
11-Sense	8047-8952	GTTGAGGCACTTTGTGA
11-Antisense		AGACACCTTCAACACCC
12-Sense	8409-9353	GAGAAACACGGAAACTA
12-Antisense		TACTGAAGATCACACCC

TABLE 2. SEQUENCES OF SECOND STAGE *ATM* PRIMERS

Fragment Number	Bases Amplified	Primer Sequence
1-T7	37-790	TAATCGACTCACTATAGGG
		CGGTTGATACTACTTTG
1-SP6		ATTTAGGTGACACTATAGAA
		TTTAATCCGTCAGTCT
2-T7	755-1632	TAATCGACTCACTATAGGA
		ATAATTCATGCTGTTAC
2-SP6		ATTTAGGTGACACTATAGAA
		TTTTATTCCAGAGTTT
3-T7	1570-2425	TAATCGACTCACTATAGGA
		AGTTGCATTGTGTCAAG
3-SP6		ATTTAGGTGACACTATAGGA
		GTTGGCTTTCTGGAA
4-T7	2368-3247	TAATCGACTCACTATAGGG
		CTGCTACTGTTACA
4-SP6		ATTTAGGTGACACTATAGAA
		TGCTCCAATTACTGT
5-T7	3055-3944	TAATCGACTCACTATAGGA
		GAAGAGTACCCCTTGC
5-SP6		ATTTAGGTGACACTATAGGA
		GATGTGGAATCAAAACCTTAT
6-T7	3911-4796	TAATCGACTCACTATAGGA
		CACAAATATTGAGGAT
6-SP6		ATTTAGGTGACACTATAGAA
		GTCCAATACCTGTT
7-T7	4782-5644	TAATCGACTCACTATAGGG
		TGGAGGTTCAGA
7-SP6		ATTTAGGTGACACTATAGAA
		GAATTTCACATTTTGT
8-T7	5607-6500	TAATCGACTCACTATAGGA
		AAGACACTGACTTGTG
8-SP6		ATTTAGGTGACACTATAGAA
		CTGCATATTCCTCC
9-T7	6451-7285	TAATCGACTCACTATAGGA
		ATAAAGACTGGTGTCC
9-SP6		ATTTAGGTGACACTATAGAA
		TTTCCAGCAACTTC

10-T7	7213-8113	TAATCGACTCACTATAGGG
		CAACTGGTTAGC
10-SP6		ATTTAGGTGACACTATAGAA
		TATTTATGCCTTTTCT
11-T7	8082-8669	TAATCGACTCACTATAGGA
		AACTTAGATGCCACTC
11-SP6		ATTTAGGTGACACTATAGGA
		AAACTGGTTGAA
12-T7	8598-9328	TAATCGACTCACTATAGGA
		AAATGATGGAGGTGC
12-SP6		ATTTAGGTGACACTATAGAA
		TCCTGGGAAAAGTCG

The underlined regions indicate the phage promoter sequences. Generally, the first two bases at the 3' end of the promoter overlapped in each case with the first two bases of the ATM fragment.

CONCLUSIONS

The first year of this study was primarily devoted to developing the technical aspects of this project so that there are few conclusions which can be reached at this time. However, the lack of *ATM* mutations in the 29 unilateral breast cancer patients examined so far are consistent with the conclusion that *ATM* heterozygosity does not play a role in susceptibility to non-radiation induced breast cancer.

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